ISOLATION AND RESTRICTION ANALYSIS OF THE CONJUGATIVE PLASMID FP2 OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Palmer, B.R, Mercer, A.A, Healy, J.P & Loutit, J.S. (1993). Isolation and restriction analysis of the conjugative plasmid FP2 of *Pseudomonas aeruginosa*. *New Zealand Natural Sciences* 20:55-61.

Physical data about the *Pseudomonas aeruginosa* conjugative plasmid FP2 has been scarce due to difficulty in isolating the plasmid's DNA. Here we describe a protocol for the preparation of moderate amounts of pure FP2 DNA. The restriction patterns obtained from digestion of FP2 DNA with four different restriction endonucleases, an improved size estimate for the plasmid and construction of an FP2 gene library in the *Escherichia coli* vector pUC19 are reported.

KEYWORDS: plasmid - restriction - cloning - gene library - *Pseudomonas aeruginosa*.

INTRODUCTION

The transfer of chromosomal genes in *Pseudomonas aeruginosa* was first reported in the 1950s following the discovery of a conjugation system (Holloway 1955 & 1956) and a transduction system (Loutit 1958). The conjugation system was found to be directed by the plasmid FP2 (Holloway & Jennings 1958). FP2 has since been classified as the sole member of the plasmid incompatibility group P-8 (Bradley 1983). FP2 has been widely employed in the mapping of markers on the *P. aeruginosa* chromosome because of the high frequency at which it mobilizes chromosomal genes. As well as chromosome mobilization ability (Cma) FP2 has been shown to code for resistance to Hg^{2+} ions (Loutit 1970) and has an estimated size of 90 kb (Pemberton & Clark 1973). $FP2^{+}$ strains have been shown to produce pili (Bradley 1983). The plasmid can be cured using mutagenic agents (Loutit 1969b, Stanisich & Holloway 1969a) or cold treatment of competent cells (Potter & Loutit 1983).

Originally the FP2 mediated conjugation system was thought to be very similar to that of F in *Escherichia coli*. Subsequent studies have revealed significant differences (Stanisich & Holloway 1969b, Wards 1986). Transfer of chromosomal markers occurs primarily from a major origin (Booker & Loutit 1974) which maps between 71 and 75 minutes on the 75 minute map (Holloway & Zhang 1990). Recombinant formation varies from as high as 10^{-2} per donor cell for the proximal *ilvB112* marker to less than 10^{-5} per donor cell for the more distal *leu-1* marker (Loutit & Marinus 1968, Loutit 1969a). At least one minor origin has been identified near *proB* (Loutit 1969a, Royle *et al.* 1981). Transfer from this secondary site appears to occur in a direction opposite to that from the major site and with lower frequency (Royle *et al.* 1981, Soldati *et al.* 1984, Wards 1986). Unlike the F fertility system the early transfer of FP2 and its lack of linkage to chromosomal markers suggest that transfer is not the result of Hfr donor formation (Loutit *et al.* 1968, Stanisich & Holloway 1969b, Wards 1986).

The isolation of FP2 DNA has proved difficult and has impeded the study of the plasmid. Despite reports of the isolation of FP2 DNA (Palchaudhuri & Chakrabarty 1976, Mercer & Loutit 1979, Potter 1985) no physical study of the plasmid has been published. Here we describe an isolation method for the preparation of FP2 DNA and the restriction patterns of FP2 DNA generated with several enzymes, steps which have led to the construction of an FP2 gene library.

METHODS

BACTERIAL STRAINS

P. aeruginosa strain OT302, a derivative of *P.aeruginosa* PAO (Loutit *et al.* 1968, Loutit, 1969a), was used for all FP2 isolation experiments. *E. coli* strain JM109 (Yanisch-Perron *et al.* 1985) was used as a host strain for cloning experiments with the plasmid vector pUC19 (Yanisch-Perron *et al.* 1985).

ISOLATION OF FP2

The isolation method used was a modification of those of Potter (1985) and Wards (1986). Luria broth (500 ml in a 2 L flask) was inoculated with 1 ml of a stationary phase Brain Heart nitrate broth culture of OT302 and incubated in a 37°C incubator with shaking for 16 h. The cells were harvested and resuspended in 20 ml of TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) and transferred to a 1 L flask. Three hundred ml of freshly made lysis buffer (TE, 4% SDS, 0.1 M NaOH pH 12.45, preheated to 55°C) was quickly added and gently dispersed. Following 20 min incubation at 55°C 30 ml of 2 M Tris pH 7.0 and 40 ml 5 M NaCl was added. The lysate was transferred to 250 ml centrifuge bottles and incubated on ice overnight. The precipitate which formed was removed by centrifugation (16, 000 g, 20 min, 4°C) and the supernatant was made 10% (w/v) with PEG 6000 and incubated overnight at 4°C before centrifugation (2000 g, 3 min, 4°C). The pellet was resuspended in 8 ml TE buffer and plasmid DNA was purified by CsCl density gradient centrifugation. After recovery of the plasmid band and ethidium bromide removal, CsCl was removed by 3 ultrafiltration steps (3000 g, 3 x 20 min) through a Centricon 30 microconcentrator (Amicon). Prior to each ultrafiltration step 1 ul of diethyl pyrocarbonate (DEP) was added. After the first 2 ultrafiltration steps the concentrated sample was diluted to its original volume with TE buffer. Following the final ultrafiltration step 5 μl of 1 m Tris pH 8.0 was added to neutralise the acidic products of DEP breakdown (Maniatis *et al.* 1981).

RESTRICTION ENDONUCLEASE DIGESTION OF FP2 DNA

Restriction endonucleases were purchased from Boehringer Mannheim (NZ) Ltd or Amersham Australia Pty Ltd and used as specified by the manufacturer.

ELECTROPHORESIS

DNA fragments from 0.5-50 kb were separated in a 1% agarose gel in 0.5 x TAE buffer at 14°C using a 9 V cm^{-1} voltage gradient at a 106° included angle with a switch time throughout of 0.40 s and a run time of 2 h in a Bio-Rad CHEF Mapper™. This program is referred to as the 0.5-50kb program.

Gels were stained for 20 mins in a solution of 1 μg ml^{-1} ethidium bromide, and destained for 1-3 hours in distilled H_2O. Gels were photographed while illuminated by an Ultra-violet Products, Inc. UV transilluminator with Polaroid 667 film in a Polaroid MP4 Land camera.

FP2 GENE LIBRARY CONSTRUCTION

DNA cloning and *E. coli* transformation were performed as described by Maniatis *et al.* (1981). Transformant colonies were screened on LAX agar (LB, 50 ug ml^{-1} ampicillin [Ap_{50}], 0.0016% X-gal) plates or MacConkey Ap_{50} plates. Putative transformant colonies were screened for plasmid content using the rapid boiling method of Holmes & Quigley (1981).

RESULTS

ISOLATION OF FP2

Two major problems encountered in purifying FP2 DNA were efficient separation of the plasmid from cell material during production of a cleared lysate and degradation of FP2 DNA after CsCl gradient purification. In the isolation procedure outlined here the first problem was resolved by using an alkaline lysis step at elevated temperature and an unusually large volume of lysis buffer. Rapid heating of the lysate to 55°C appears to improve yield and purity of FP2 DNA using this procedure. Degradation of purified FP2 DNA was avoided by the combined use of microconcentrators and DEP.

The plasmid isolation method used yielded 5-10 μg of CsCl-purified FP2 DNA (determined spectrophotometrically) from a litre of stationary phase culture. Care was taken in the isolation procedure to ensure that early stationary phase cells were used. The pH of the lysis mixture was rigourously checked, it must be between pH 12.4-12.5. The addition of DEP, as a nuclease inhibitor (Ehrenberg *et al.* 1976,

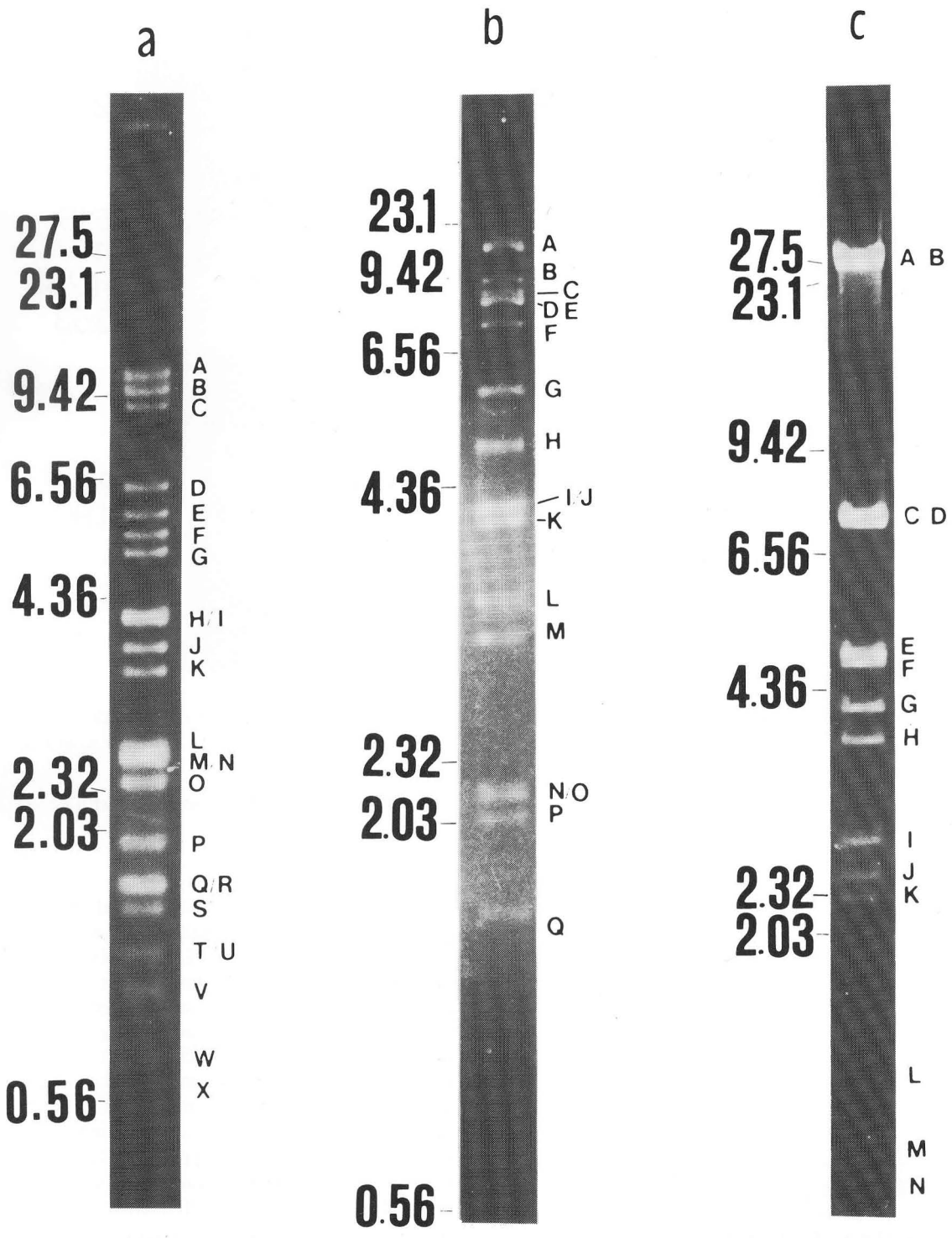

Figure 1. Restriction endonuclease digest of CsCl-purified FP2 DNA with a) *BglII*, b) *EcoRI*, c) *HindIII*. Size standard marker mobilities are indicated with fragment sizes in kb.

Maniatis *et al.* 1982), was also essential following the removal of CsCl from gradient-purified FP2 DNA. In the absence of DEP the DNA degrades in less than 16 h at 4°C.

Restriction analysis

FP2 DNA was digested with the enzymes *Bgl*II, *Eco*RI and *Hind*III to give the restriction patterns shown in Fig. 1. Size estimates of these restriction fragments are given in Table 1. This indicates an approximate size of 93 kb for FP2. Double digests of FP2 DNA with the three enzymes used above in combination with the rare cutter *Xba*I suggest FP2 contains at least three *Xba*I recognition sites. Digests of FP2 with *Xba*I alone yielded variable restriction patterns. A double digest of FP2 with *Eco*RI and *Xba*I is shown in Fig. 2.

Table 1. Sizes of FP2 Restriction Fragments[1]

Fragment	*BglII* (kb)	*EcoRI* (kb)	*HindIII* (kb)
A	11.0	14.2	26.8
B	10.0	9.4	26.8
C	9.2	8.8	7.5
D	6.5	8.3	7.5
E	5.9	8.3	4.7
F	5.5	7.3	4.5
G	5.2	5.6	4.1
I	4.2	4.2	2.8
J	3.8	4.2	2.5
K	3.5	4.0	2.3
L	2.8	3.3	1.1
M	2.6	3.1	0.9
N	2.6	2.2	0.7
O	2.4	2.2	
P	1.9	2.0	
Q	1.7	1.6	
R	1.7	0.9	
S	1.5	0.6	
T	1.3	0.3	
U	1.3		
V	1.1		
W	0.9		
X	0.7		
Total Size	91.6	93.1	95.9

[1]The size of each fragment is a mean value determined from at least different two estimations (using the computer program Gels (Buckler, 1985)) performed on data from two different AGE gels.

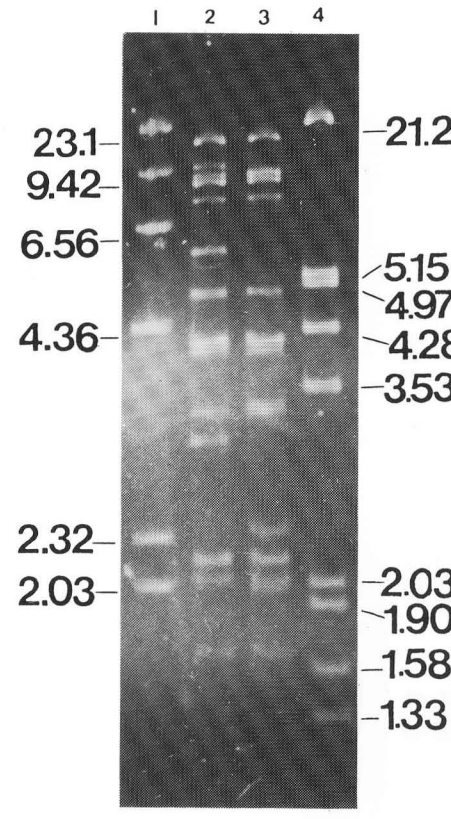

Figure 2. Double digest of FP2 with *Eco*RI and *Xba*I. Lane 1 Lambda *Hind*III. Lane 2 FP2 *Eco*RI. Lane 3 FP2 *Eco*RI/*Xba*I. Lane 4 Lambda *Eco*RI/*Hind*III.

Gene library construction

*Eco*RI fragments of FP2 were ligated to *Eco*RI cut pUC19 before transformation into competent JM109 cells and screened on LAX or MacConkey Ap_{50} plates. Colonies containing insertionally inactivated pUC19 were screened for insert size and the total array of unique fragments cloned is shown in Fig. 3. One previously undetected *Eco*RI fragment was identified at this stage with a size of approximately 300 bp. As the *Eco*RI digestion pattern of FP2 contains three double bands (fragments D & E, I & J and N & O) plasmids containing cloned fragments corresponding to these sizes were digested with *Eco*RI and *Hind*III in order to identify the different insert fragments. This strategy allowed the identification of two fragments with different restriction patterns for each doublet pair. All *Eco*RI

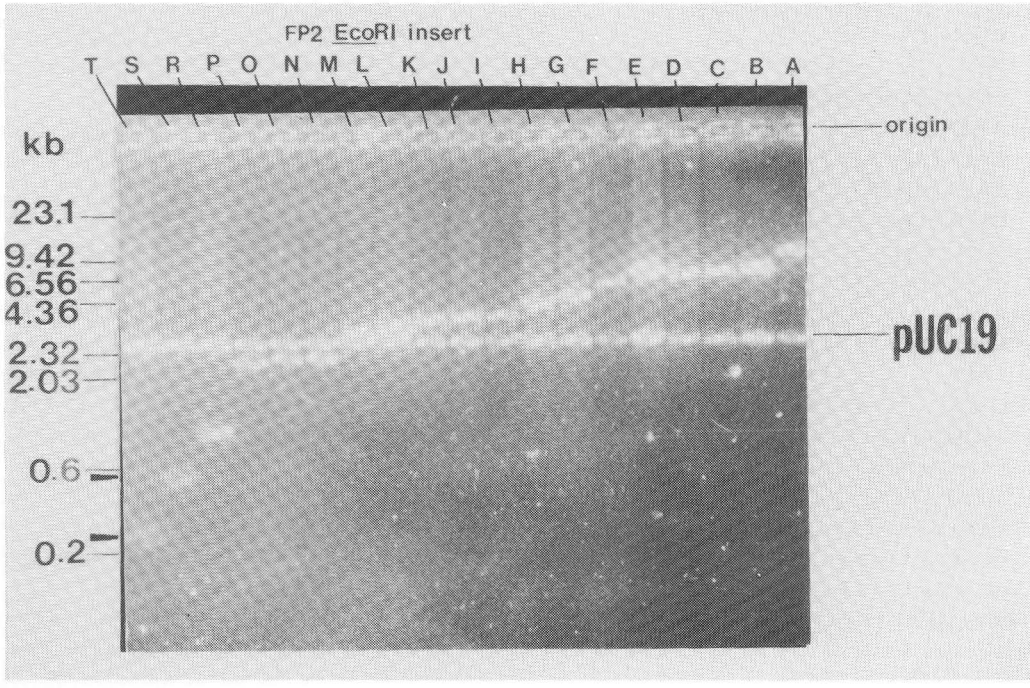

Figure 3. Size ordered *Eco*RI FP2 clones released from pUC19 by digestion with *Eco*RI. Recombinant pUC19 clones of FP2 were digested to completion by *Eco*RI and anaylsed by AGE using the 0.5 - 50 kb program of the CHEF Mapper (see methods). The 1.6 kb *Eco*RI Q fragment has not yet been cloned.

fragments of FP2, with exception of 1.6 kb *Eco*RI Q, have been successfully cloned. Directed cloning of FP2 *Eco*RI Q has so far been unsuccessful.

DISCUSSION

The FP2 isolation method outlined here has allowed the purification of sufficient FP2 DNA for a physical study of the plasmid to be undertaken and the construction of a gene library of the plasmid. The restriction endonucleases used produce a relatively large number of restriction fragments, with the exception of *Xba*I which recognises at least three but probably no greater than five sites on FP2. Digestion of FP2 with *Eco*RI and *Xba*I revealed three *Eco*RI fragments are cut by *Xba*I. Fewer *Xba*I cuts were visible in double digests with *Hind*III and *Bgl*II presumably because some *Xba*I sites lie close to sites for these enzymes. Relatively few sites for *Xba*I on the *P. aeruginosa* chromosome have also been observed (Römling *et al.*, 1989), presumably due to the presence of the rare tetranucleotide CTAG in the recognition site. Reproducible restriction digests of FP2 DNA have allowed size estimations of the entire FP2 genome. An overall size of approximately 93 kb for FP2 is indicated and this agrees well with the findings of Pemberton and Clark (1973).

The gene library constructed contains an almost full complement of *Eco*RI fragments of FP2 as seen in complete digests of the plasmid. Potentially, fragments containing replication origins may be unstable and thus be difficult to isolate as stable clones. Also some fragments may encode genes that result in the expression of lethal products in an *E. coli* background. Work on a complete restriction map of FP2 and on characterising FP2-encoded genes is continuing. The FP2 gene library represents a valuable resource for use in achieving both of these goals and for further studies of the plasmid.

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